Original Article

Tissue Engineering Using Differentiation of Endometrial Stem Cells into Ameloblast Cells and Implantation on Hydroxyapatite and Collagen Nanocomposite Scaffold

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Abstract

Background: Dental tissue engineering (DTE) is an inspiring biological approach to replacing lost teeth. The strength of the differentiation of basic endometrial cells to ameloblasts is yet to be fully understood. Moreover, the construction of a scaffold, upon which ameloblasts can both grow and replicate, has been a challenge in DTE. **Methods:** This is an experimental study. Basic endometrial cells were first isolated and cultured before induction of differentiation to ameloblast cells by adjacency to mesenchymal rat cells and application of fibroblast growth factor 8. Hydroxyapatite nanoparticles were synthesized by freeze-drying method. The nanocomposite collagen and hydroxyapatite scaffold were synthesized by the solvent casting method. Finally, the amyloblast cells were implanted into the hydroxyapatite and collagen nanocomposite scaffold. **Results:** The success of cell differentiation was confirmed through staining using specific antibodies against ameloblastin and amelogenin markers. Expression of ameloblast-specific mRNAs, such as ameloblastins, amelogenins, and cytokeratin 14 was detected in the differentiated cells. Alizarin red staining clearly illustrates mineralization nodules in the differentiated cells. Staining against specific ameloblast markers showed that the differentiated ameloblast cells are expressed on the scaffold, unlike the control group. **Conclusion:** Based on the findings of this study, basic endometrial cells are capable of expressing the traits of ameloblast cells. The scaffold used in the present study conforms to most criteria used for dental tissue reconstruction and can be applied in DTE.

Keywords: Ameloblast cells, collagen nanocomposite, dental tissue engineering, endometrial stem cells, hydroxyapatite, scaffold

Introduction

Replacement of lost enamel relies on the use of artificial regenerative materials, such as polymers, metals, and ceramics, which are mostly lost due to weak adhesion and/or cracking. Dental tissue engineering (DTE) is a multidisciplinary field of biological and engineering principles and applications that basically aims to identify the relationship between the structure and performance of natural and diseased tissues. Studies show that tissue engineering could be used for the reconstruction of biological tissues.[1] Somehow, tissue engineering has been applied by dentists for centuries for dentinogenesis in the form of calcium hydroxide in pulp therapy and dental bridge formation.[2] Nowadays, tissue engineering has progressed

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toward the construction of similar dental tissues using biological techniques.[3] So far, various stem cells have been isolated from dental tissue, such as pulp stem cells, periodontal ligament stem cells, and milk teeth stem cells.[4] Determining the type of stem cells is very important in DTE. These cells can be derived from fetal or mature cells. Most of the recent efforts in this field are focused on mature stem cells mature stem cells can play important roles in dental tissue formation due to their divisional and differentiation characteristics. By far, mature dental pulp stem cells and milk teeth pulp stem cells have been differentiated into cells similar to odontoblasts.[5] Various cellular and molecular studies have shown that oral epithelium triggers the induction, formation, and growth of teeth by sending direct signals at the fetal stage. Few studies have been conducted on the success of stem cell differentiation into dental epithelial cells.[6] Apart from organ enamel cells, few cellular sources have been identified for the replacement and regeneration of ameloblasts. Based on the primary studies, dental pulp-derived stem cells, bone marrow, and fetal cells have the potential to differentiate into ameloblasts.[7] Nevertheless, one of the practical barriers in this field is the limitations in clinical sampling. An appropriate source for seeding cells to regenerate tooth enamel is still challenging and yet to be found. Recent immunohistochemical findings that illustrated the expression of various markers, particularly Oct-4, CD146, and STRO-1, have revealed the presence of stem cells in different layers of the uterus. The possibility of transforming stem cells into ameloblasts has been examined in the present study, considering ease of access to endometrial stem cells (EnSCs) (even at a late age), angiogenesis, nontumor formation, and proof of transformation of EnSCs into other cells, such as osteoblasts and odontoblasts.[8] Considering the advantageous characteristics of EnSCs over other types of stem cells, it was hypothesized that culturing EnSCs in adjacency to rat dental mesenchymal cells, together with fibroblast growth factor 8 (FGF 8), can trigger differentiation of these cells into ameloblasts. Furthermore, to improve the efficacy of cell and tissue culture in tissue engineering, *in vitro* three-dimensional (3D) conditions are required to be created.[9,10]

Methods

Ethical consideration

Ethical approval for this study was obtained from the Research Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.NRITLD.REC.1403.018).

Type of sampling and reasons for selection

Cell isolation and coculture

Endometrial biopsy samples were isolated using our previously published protocol. Briefly, the endometrial tissue was transferred to a centrifuge tube containing 15 mL collagenase type IA (2 mg/mL was solved in DMEM-containing 1% antibiotic × 100) and was then incubated at 37°C for 2 h. After tissue digestion, epithelial and stromal cells were separated using 70 μm and 45 μm filters. The separated cells were then centrifuged for 15 min at 1000 g. The cell pellet was then purified using favicol. The obtained cellular depositions were mixed with a 5 ml ordinary culture containing DMEM containing 1% antibiotic, 1% glutamine, and 10% fetal bovine serum (FBS) to form a suspension. EnSCs were transferred to a culture flask which was then incubated at 37°C, 5% CO_2, and 95% humidity. To induce differentiation, EnSCs were cultured in adipogenic and osteogenic media for 14 days as described previously.

The lower molar teeth buds of 11 rat fetuses were extracted using a scalpel. The buds were placed in 1% trypsin at 37°C for 2–3 h to allow the enzymatic separation of epithelial and mesenchymal parts of the teeth. After enzymatic digestion, the mesenchymal part of the teeth was completely separated from the epithelial part under a microscope. Thereafter, the dental mesenchymal cells were washed with saline phosphate buffer and were exposed to 1% collagenase for 3 h at 37°C to allow the cells to individualize. In the next step, they were passed through a 70 mm nylon filter. The obtained cells were transferred into a 24-well plate containing 0.5 mL of ordinary DMEM culture environment containing 1% antibiotic (×100), 10% FBS, and 1% glutamine and were then incubated at 37°C, 5% CO_2, and 95% humidity. Cells were kept in culture for a week to reach the desired density.

Inclusion criteria

After the third passage of the endometrial cells at a density of 1×10^6 cells/mL, they were transferred to a 6-well plate of insert culture containing 2 mL of ordinary DMEM culture and 10% FBS.

Exclusion criteria

Hematopoietic stem cells and cells without surface markers were excluded from the study.

In the second passage, the mesenchymal cells were seeded in the internal plates of the insert culture with a density of 1×10^5 cell/mL. After 24 h of adjacency of the two cells, their organization and changes were constantly examined using a reverse microscope. To examine mineralization and formation of calcification nodules, cells, which were kept in culture for 28 days, were stained with 2% alizarin (pH = 4.2–4.4) for 30 min at 37°C.

Immunostaining

The differentiated cells, which were kept in culture for 14 days, were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich) for 20 min at RT. Furthermore, 0.1% TX-100 in TBS was used to permeabilize the cells which were then blocked with 5% bovine serum albumin (BSA) and incubated overnight with primary antibodies against ameloblastoma (mouse monoclonal antihuman; Abcam, USA, 1:200) and amelogenin (mouse monoclonal antihuman; Abcam, 1:200). Secondary antibody included Alexa Fluor 488 donkey antimouse (1:500; Gibco, A-11058) and the nuclei were counterstained with DAPI (Sigma-Aldrich, D8417).

Real-time – polymerase chain reaction

Total RNA was extracted using a Rneasy PLUS mini kit (Sinaclone/Iran). RNA concentration and purity were assessed by measuring 260/280 nm absorbance on a nanospectrophotometer. Isolated RNA with a 260/280 ratio of ~ 2 was used for further experiments. Isolated RNA (1 μg) was reverse transcribed to cDNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas, USA). Gene expression was determined by real-time polymerase chain reaction (PCR) using SYBR green. Since it has been reported that GAPDH is the most stable housekeeping gene, it was used as the internal comparator in parallel with the control sample. Primers were validated before being used for experiments. PCP reaction mixture was prepared using 0.1 μM of each forward and reverse primers, whereas 300 nM of each primer was used in the real-time PCR reaction mixture. Relative gene expression was analyzed using Step One software V2.0 (Applied Biosystems; Foster City, CA, USA), and the baseline and threshold were set manually. Reverse transcription PCR data were analyzed using the ΔΔCt method.

Scaffold preparation

To prepare hydroxyapatite, solutions containing calcium and phosphorus – separately – were used, along with calcium nitrate tetrahydrate ($Ca[NO_3]_2.4H2O$]) and diammonium phosphate ($[NH_4]_2HPO_4$). Distilled ionized water was used to prepare water mixtures of collagen-hydroxyapatite. The next procedure was freeze-drying the samples. Since the goal of this procedure was to build a 3D scaffold, the prepared layers were cut into smaller pieces and attached to each other using a 10% collagen solution. The samples were inserted in a glutaraldehyde ($C_5H_8O_2$) solution to increase the strength of the composite scaffold.

Compression strength

To perform this test, three synthesized scaffolds were selected using the aforementioned method and prepared in cylinder form of 1 cm height to 0.5 cm diameter. Before performing the test, the diameter and height of each cylinder were measured at three distinct regions and the mean diameter and height were calculated. The compression strength of the scaffold was measured according to the ASTM F 451-86 standard, using the Mechanical GmbH and CO. KG, Germany Zwick/Roell Z020, Zwick device, at a speed of 0.5 mm/min.

The scaffold toxicity test

The MTT assay was performed to examine the toxicity of the scaffold. After determining the degree of light absorbency against the number of relevant cells, the standard curve was drawn.

Culturing ameloblasts on the nanocomposite scaffold

The scaffold was first sterilized with ultraviolet rays for a minute before slowly pouring 9 cc DMEM containing 10% FBS onto the nanocomposite which was then incubated at 37°C for 45 min. The differentiated ameloblast cells (density = 10^6 cells/mL) were then poured onto the composite which was then incubated at 37°C, 90% humidity, and 5% CO_2 for 48 h. Amelogenin and ameloblastin antibodies were used to perform immunohistochemistry.

After 14 days of induction, cells were fixed with 4% PFA, Sigma-Aldrich for 20 min at room temperature and then permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. The nonspecific binding sites were blocked for 30 min at room temperature with 5% BSA and incubated with primary antibodies against amelogenin (1:200, mouse monoclonal antihuman, Abcam), and ameloblastin (1:200, mouse monoclonal antihuman, Abcam) diluted in 5% BSA in PBS overnight. Secondary antibodies included Alexa Fluor 488 donkey antimouse (1:500, Gibco, A-11058). The nuclei were counterstained with 1.5 μg/mL DAPI (Sigma-Aldrich, USA). For negative controls, only the secondary antibodies were used. The positive markers were evaluated using Olympus DP70 fluorescent microscope (USA).

Results

Cell isolation and characterization

Flow cytometric analysis showed that EnSCs were positive for mesenchymal stem cell markers, such as CD90, and were negative for hematopoietic cell marker CD34. To prove that endometrial cells are a type of stem cells, the osteogenic and adipogenic differentiation were investigated The third passage cells that had been kept in the osteogenic medium developed the following morphological features: after adding the differentiating environment, the cells became shorter, and more cubical in shape and began to secrete mineral matrix, which turned red upon staining with alizarin, resulting from the mineralization of the secreted matrix. Moreover, after 3 weeks of endometrial cell differentiation in the adipogenic environment, the cells changed morphologically and became cubic. Gradually, lipid vacuoles developed in them, and oil red staining showed the accumulation of lipid droplets in the cellular cytoplasm [Figure 1].

Ameloblast cells differentiation of endometrial stem cells analysis

Examination of the differentiated stem cells during the study showed that cells in the control group did not change morphologically, 14 days after induction and that the characteristics of undifferentiated cells were preserved, i.e. they were rich in cytoplasm and countless additives. The results of ameloblast markers expression showed that hEnSCs can differentiate into ameloblast cells as the result of interaction with dental mesenchyme cells. However, the cells in the differentiating medium became polarized. Mineralization nodules were clearly seen after 14 days of coculturing with dental bud mesenchyma and FGF8, using alizarin red staining. On the other hand, mineralization nodules were not formed in the control group [Figure 2]. Immunostaining against specific ameloblast markers showed that ameloblastin and amelogenin cell markers were expressed after 14 days of treatment. This expression, however, was not observed in the control group [Figure 3a]. Results of mRNA analyses showed that

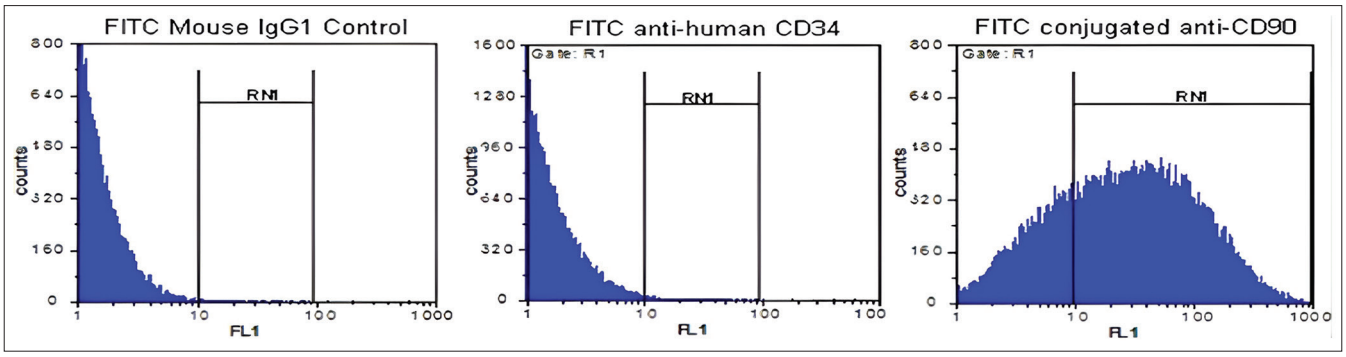

Figure 1: Flow cytometric analysis for CD90 and CD34

ameloblastin, amelogenin, and cytokeratin 14 were expressed in both the control and experiment groups that was treated with differentiating factors for 14 days. Ameloblast results mRNAs of cell markers including amelogenin, ameloblastin, and cytokeratin 14 with control showed that cells treated for 14 days with differentiation factors were the markers expressed mRNAs [Figure 3b].

Scaffold testing

The tested samples (cylinder shaped) became 50% shorter and the pressure tolerance. The compression strength was 2.045 MP [Figure 4]. The MTT results showed a 65% survival rate of cells during the 48 h of adjacency.

Electronic microscopy micrographs demonstrated that cells attached grew and spread on the hydroxyapatite and collagen nanocomposite scaffolds [Figure 5]. These results showed that these scaffolds might be a kind of potentially appropriate scaffolds for the differentiation of EnSCs ameloblast cells.

Discussion

In the present study, EnSCs have been applied as a new source for DTE and dental enamel production. To the best of our knowledge, this is the first study that investigated the differentiation of EnSCs into ameloblast cells. EnSCs are one of the different types of mesenchymal cells. The first published study that proved the presence of endometrial stem and progenitor cells was performed on human clonogenic epithelial and stromal endometrial cells.[11] Researchers have suggested that the high regeneration capability of human endometrium is due to the presence of a collection of stem cells in this tissue.[12] Studies have shown that the thickness of the human endometrial wall grows from 0.5–1 to 5–7 mm after each menstruation, at the end of the menstrual cycle. The generation of ameloblast cells from EnSCs has the potential to be applied in DTE, dentistry, cell therapy, etc., Cellular interactions, differentiation-inducing factors or factor, and duration of induction are some of the important factors affecting the differentiation of embryonic stem cell (ESC)-derived ameloblast cells. In this study, cells began to create specific colonies around individual cells, from the very 1st days, when they were cultured in the culture flask.[13] Furthermore, the results of flow cytometry, which was used to identify the mesenchymal stem cell markers, showed that CD105, CD90, and OCT4 were positive and CD31, CD14, CD61, and CD34 were not expressed in these cells.[14] In the present study, the accurate cell count and flow cytometry analysis showed that 98.98% of cells positively expressed CD105, a percentage that is relatively higher than that reported by previous studies. Moreover, the percentage of cells that positively expressed CD90 (71.17%) was in line with that found in previous reports. In the current study, since the cell population was very heterogeneous on the 1st few days of isolation, flow cytometry analysis was not performed on the cells on the 1st day of isolation. The amelogenetic capability of ESC was measured in this study, during a 14-day period of adjacency to fetal rat dental mesenchymal cells along with FGF8. The expression of specific ameloblast genes, including amelogenin, ameloblastin, and cytokeratin 14 was then studied using quantitative real-time PCR. The protein expression of amelogenin and ameloblastin was also investigated using immunocytochemistry and immunohistochemistry. They have shown that bone marrow cells could be induced to be differentiated to both ameloblasts and odontoblasts in the adjacency to epithelial and mesenchymal cells. The results of the current study showed that endometrial cells undergo morphologic changes, i.e. cell polarization as well as increased cellular components, in adjacency to rat mesenchymal dental cells, and FGF8. On the other hand, cells in the control group became elongated and fusiform. A research group has investigated the differentiation properties of three key growth factors including Bone Morphogenetic Protein 4 (BMP4), Serum sonic hedgehog (SHH), and Fibroblast growth factor 8 (FGF8) inducing the differentiation of keratinocyte stem cells into human ameloblasts.[9] Among these three factors, SHH and BMP4 were expressed in human and mouse ameloblasts. On the other hand, FGF8 was expressed in both ameloblasts and odontoblasts, in the initial and final stages of human teeth development.[15] Neither SHH nor BMP4 were capable of inducing ameloblastic differentiation. Moreover, BMP4 induced stem cell differentiation into bone, instead of teeth. Whereas, keratinocyte-derived stem cells were differentiated into ameloblasts in the presence of FGF8, such that they were elongated and began to secrete enamel. Among the odontogenic signals, FGF8 seems to be the most vital. In

mice, FGF8 is expressed in the dental epithelium at the onset of development and is responsible for Pix2 induction, which interferes with dental epithelial characteristics.[15,16] Using *in vitro* stem cells (dental pulp stem cells, periodontal ligaments stem cells, and apical papilla stem cells) for human DTE and regeneration will have an extraordinary effect on the history of dentistry. A couple of recent studies have identified EnSCs as a natural source of odontoblasts. They have shown that in suitable *in vitro* media, cells can differentiate into odontoblasts and secrete dentinal proteins.[16,17] According to these findings, mesenchymal-originated cells have the ability to differentiate into two types of dental cells with two different fetal origins: odontoblasts with mesenchymal origins and ameloblasts with ectodermal origins. These findings as well as the findings of the present study can be considered promising developments in complete DTE. In this study, a nanocomposite collagen and a hydroxyapatite scaffold were used to seed cells. With respect to the chemical combination and structure, hydroxyapatite is similar to the nonorganic part of bone; hence, hydroxyapatite is biocompatible with soft and hard tissues. In other words, despite being osteophilic, it is not absorbed and it does not disintegrate. It stimulates osteogenesis as well as dentinogenesis and triggers bone growth within the existent porosities (the porosities present between the granules or pieces). Hydroxyapatite creates a direct connection with the bone and forms a desirable adhesion.[18] Therefore, considering the biocompatibility properties of hydroxyapatite with bone and soft tissues, it is potentially the best substitute for the hard tissues of the body. Collagen is also the most abundant present proteinous-fibrous tissue inside the human body and other creatures which exists in various body parts, such as skin, connective tissue, neuronal,

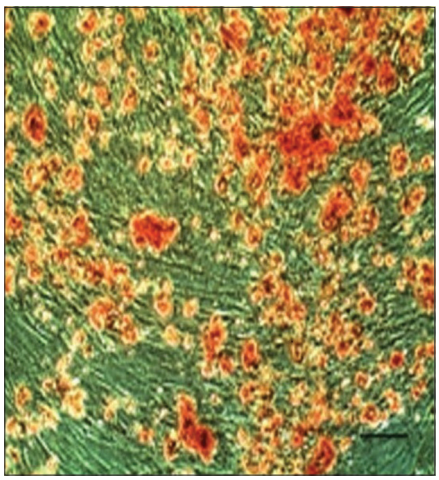

Figure 2: Alizarin red staining after 14 days of coculture of endometrial cells and bud with fibroblast growth factor 8

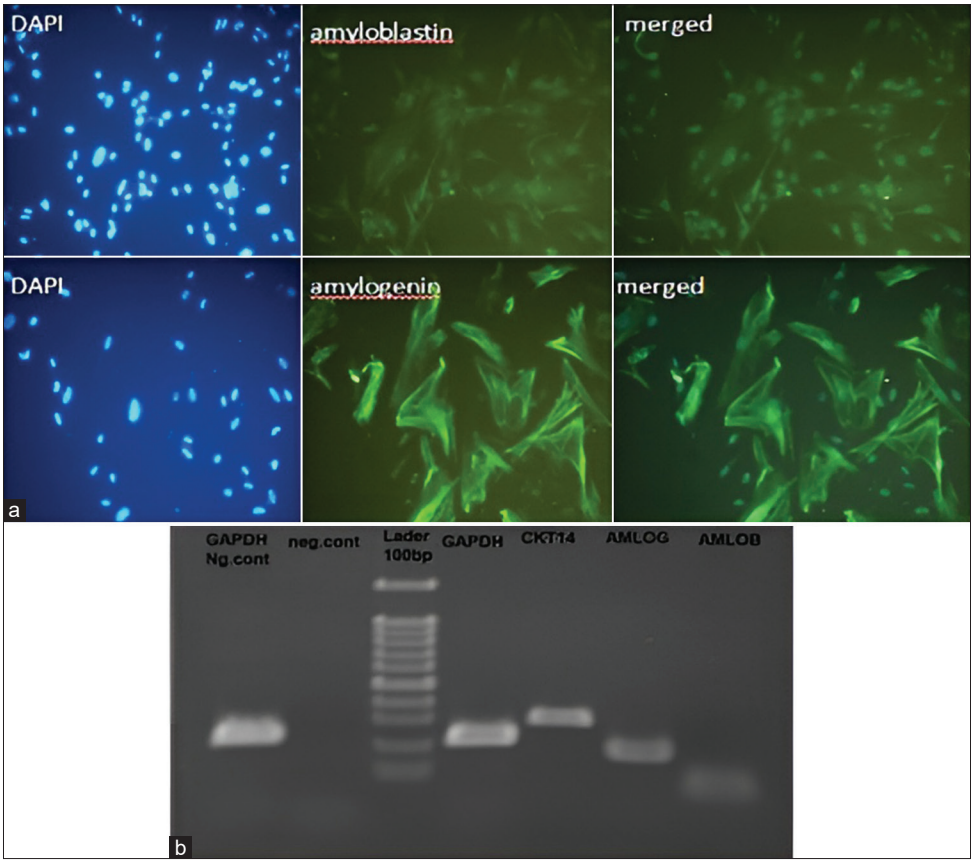

Figure 3: Gene expression analysis. (a) Expression of ameloblastin marker and amelogenin. Immunohistochemistry analyses showed no expression of both markers in the control group. (b) Reverse transcription-polymerase chain reaction analysis for expression of amelogenin, ameloblastin, and cytokeratin 14 compared to the control, after 14 days of culture

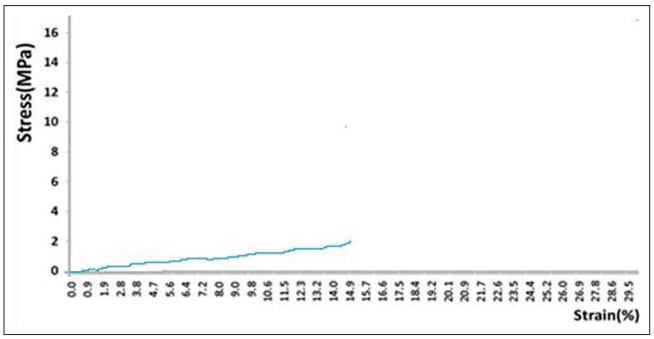

Figure 4: Compressive strength graph scaffolding cross-linked with glutaraldehyde 4%

and dental cells. The combination of these two materials can create a structure similar to that observed in teeth.[19,20] The lifespan of odontoblasts on this scaffold was approximately 54%, which was somewhat similar to that observed in the present study.[21] In addition, they have used the treated dentin matrix to restore tooth roots and can successfully induce the growth of odontoblasts.[22] In another study, a hybrid scaffold, polycaprolactone-poly (glycolic acid), was used to repair dental ligament tissue. The results of histological studies showed that bone formation and density have been done.[23,24] Also, a collagen-hydroxyapatite-PLA nanocomposite scaffold was used, and the results of histological studies showed new bone growth, which is consistent with the findings of this study.[25]

Conclusions

Based on the findings of the present study, EnSCs are capable of exhibiting characteristics of ameloblast cells. The ability of these cells to differentiate into ameloblasts in adjacency to dental mesenchymal cells indicates the significance of interactions and signaling between epithelial and mesenchymal tissues, and accordingly the utilization of this approach in DTE. EnSCs can definitely be considered a suitable source for DTE and be used in dental tissue restoration or regeneration. The scaffold used in this study meets most of the essential criteria to be applied in dental tissue regeneration.

Limitations of the study

The main challenge faced in this study was the lack of materials including the required antibodies and kits.

Outcome of the study

The results of other studies on the differentiation of these stem cells into ameloblasts as well as the findings of the present study indicate that it has the potential to be used in the development of complete DTE and the treatment of toothless, caries, and diseases of the elderly.

Rational of the study

To achieve this goal, cells were cultured into a scaffold similar to the structure of the teeth (a combination of hydroxyapatite and type I collagen). The cellular activity of the ameloblasts was then examined considering the existing standards.

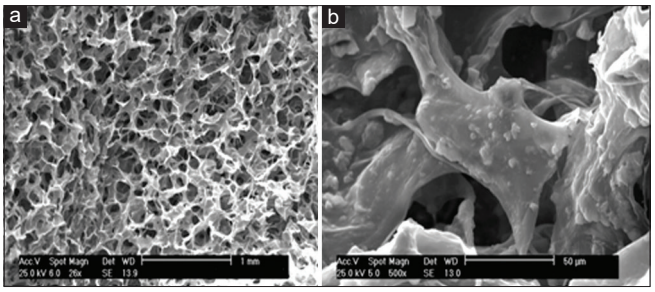

Figure 5: Scanning electron micrographs showing nanocomposite collagen and hydroxyapatite scaffold with and without cells. (a) The fibers of the scaffold were randomly entangled to form a strong and porous three-dimensional matrix. (b) Endometrial stem cells cultured on nanocomposite collagen and hydroxyapatite scaffold that grew on this scaffold 7 days after seeding (scale bar 50 μm)

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Nil.

Conflicts of interest

There are no conflicts of interest.

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